

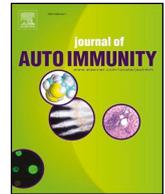
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Autoantibodies to neutrophil extracellular traps represent a potential serological biomarker in rheumatoid arthritis



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ABSTRACT

Neutrophil extracellular traps (NETs) are networks of extracellular chromatin decorated with antimicrobial proteins, formed by neutrophils to entrap pathogens. NETs have been implicated in the generation of autoimmune reactions. Here, we investigate the reactivity of rheumatoid arthritis (RA) serum antibodies with NETs and explore whether anti-NET antibodies (ANETA) have a potential as biomarker in RA. To quantify ANETA, we developed an ELISA with NETs isolated from stimulated human neutrophils and verified the results by immunofluorescence staining of NETs. ANETA were detected in 22%–69% of RA sera. No significant differences were observed in the reactivity of RA sera with NETs originating from RA patients and healthy control neutrophils, nor with NETs induced by phorbol 12-myristate 13-acetate or the calcium ionophore A23187. ANETA were detected already at baseline in newly diagnosed RA patients and both increased and decreased levels were observed in samples with a median follow-up of 7 years. By ANETA ELISA, we showed that ANETA are also present in sera of patients with systemic lupus erythematosus (36%), Sjögren's syndrome (76%) and scleroderma (61%). In addition to antibodies to NETs, also the presence of NETs or NET fragments in RA sera was determined using a sandwich ELISA. Elevated levels of NETs or NET fragments were detected in 32% of the sera. To assess the potency of ANETA as a biomarker in RA, we compared ANETA positivity with other clinical features. The presence of ANETA was significantly higher in rheumatoid factor (RF)-positive patients, but did not correlate with anti-citrullinated protein antibodies (ACPA), nor with the presence of NET fragments in serum. In addition, no correlation was observed with age, gender, onset of the disease, disease activity and inflammatory markers. These findings suggest that ANETA may be an independent biomarker in RA and possibly also in other autoimmune diseases.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease, which affects about 1% of the world's population and is characterized by inflammation-mediated joint destruction [1]. Two autoantibody reactivities that are frequently found in the sera of RA patients, anti-citrullinated protein antibodies (ACPA) and antibodies against the Fc portion of IgG, named rheumatoid factor (RF), are included in the ACR/EULAR criteria for the classification of RA [2]. The presence of these antibodies correlates with disease activity, prognosis and outcome [3]. Both ACPA and RF are sensitive biomarkers for RA, although RF is less specific [4]. A relatively small, though significant group of RA patients have neither ACPA nor RF (seronegative RA) and are being diagnosed via physical

examination, imaging of the affected joints, or by other means. The identification of new biomarkers for RA that facilitate diagnosis, have prognostic potential for disease outcome or are predictors for treatment response, is an ongoing challenge.

About 15 years ago, neutrophil extracellular traps (NETs) were described as a new mechanism of neutrophils to fight infections [5]. NETs consist of chromatin mixed with granular proteins and neutrophils form them to entrap pathogens. Soon after their discovery, NETs were implicated in multiple autoimmune diseases, such as systemic lupus erythematosus (SLE), ANCA-associated vasculitis and RA [6–8]. Neutrophils of RA patients were found to have an increased propensity to form NETs and to respond more vigorously to neutrophil-activating stimuli [9–11].

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Interestingly, several studies have identified citrullinated proteins on NETs, including histones, neutrophil elastase, azurocidin and myeloid cell nuclear differentiation antigen (MNDCA) [9,12,13]. These proteins may contain epitopes that are recognized by autoantibodies in RA sera. Indeed, RA sera were shown to have antibodies against NETs, and their reactivity was diminished when ACPA were depleted from the sera [9,13–15]. ACPA have also been reported to stimulate NET formation, suggesting an inflammatory feed forward loop [9,16,17]. Little is known about the other anti-NET antibodies in RA that do not target citrulline, and the overall prevalence of ANETA in RA patients is unclear.

The aim of this work was to assess the prevalence of autoantibodies against NETs in RA and their potential as a biomarker in RA. The prevalence of ANETA in RA patient sera was determined by an anti-NET ELISA with four different types of NETs and the results were verified by an immunofluorescence assay. In order to determine the clinical value of ANETA as a biomarker, the results were compared with ACPA and RF levels. Finally, we compared ANETA levels with other clinical parameters such as the presence of circulating NETs or NET fragments, disease activity scores and inflammation markers.

2. Material and methods

2.1. Patient sera

This study was carried out in accordance with the recommendations of the National Research Ethics Service (NRES) Committee Northwest (Greater Manchester West), UK for RA patients and was approved by the 'Commissie Mensgebonden Onderzoek' region Arnhem Nijmegen, with written informed consent from all subjects in accordance with the Declaration of Helsinki. All RA patients fulfilled ACR criteria for RA and were recruited from clinics at University Hospital Aintree in Liverpool and at Radboud University Medical Center/Sint Maartenskliniek in Nijmegen. The sera from RA patients at baseline and follow-up were from the Leiden Early Arthritis Clinic and have been described previously [18]. The scleroderma, systemic lupus erythematosus and Sjögren's syndrome patient sera were a kind gift from Dr. Alain Meyer (Université de Strasbourg, Strasbourg, France).

2.2. RF quantification in sera

RF was determined by ELISA (Rheumatoid Factor Ab ELISA kit; Abnova Cat. no. KA1442) in accordance with the manufacturer's instructions.

2.3. Measurement of soluble NET components in sera

The amount of NETs in the sera was quantified as has been described previously, using a NET ELISA [19]. Briefly, a sandwich ELISA was performed in which the anti-MPO antibody (Biorad Cat. no 0400-0002) served as a capturing antibody to bind NETs or NET remnants. The anti-DNA antibody conjugated to peroxidase (Cell Death Detection ELISAPLUS, Roche, Cat. no 11774425001) was subsequently used to detect bound NETs.

2.4. Neutrophil isolation and induction of NET formation

Blood was collected from healthy donors or from RA patients (with informed consent from all subjects). To 25 ml of anti-coagulated blood 5 ml HetaSep (Stemcell Technologies, Cat. no 07806) was added, followed by incubation for 30 min at 37 °C. The top layer containing white blood cells was then collected and layered on top of 15 ml Ficoll (GE Healthcare, Cat. no 17-1440-02) and centrifuged for 30 min at 500 ×g. The granulocytes were washed once with PBS and remaining red blood cells were removed by hypotonic lysis with ammonium chloride (150 mM). Subsequently, the granulocytes were collected by

centrifugation and resuspended in pre-warmed DMEM/F12 medium without phenol-red (Gibco, Cat. no 11039-021). Per Petri dish (60 cm²), 20 to 30 million neutrophils were seeded in a total volume of 15 ml. The cells were allowed to adhere to the plate for 30 min at 37 °C and subsequently stimulated with 5 nM PMA (Sigma Aldrich, Cat. no P1585) or 4 μM A23187 (Sigma Aldrich, Cat. no C7522). NETs were allowed to form during culturing for 3 h and 15 min. In order to inhibit proteolytic degradation of NET-associated proteins, PMSF was added at 2 h 15 m, 2 h 30 m, 2 h 45 m and 3 h after induction for the PMA-treated cells and at 2 h 45 m and 3 h after A23187 stimulation [20,21]. After 3 h 15 m, the culture medium was discarded and the cells were washed once with PBS before incubation with 4 ml 5 U/ml micrococcal nuclease (MNase; Boehringer Mannheim, Cat. no. 85446620) in pre-warmed DMEM/F12 medium for 10 min at 37 °C. The supernatant was collected, supplemented with 5 mM EDTA to inhibit MNase and centrifuged for 5 min at 1500 ×g to remove cell remnants. The resulting NET harvests were supplemented with 10% glycerol and 1 mM dithiothreitol and stored at –20 °C until further use.

2.5. ANETA ELISA

Multiple NET harvests originating from several blood donors, either RA patients or healthy individuals, and from neutrophils stimulated with either PMA or A23187 were pooled in order to generate homogenous NET preparations sufficient for the analysis of all tested samples. The amount of DNA was determined by adding 5 mM of Sytox Green and measuring the DNA content using a DNA calibration curve. In a Nunc Maxisorp plate (Thermo Fisher Scientific, Cat. no 442404), NET harvests corresponding to an equivalent of 675 ng DNA was added to each well (in 100 μl DMEM) and incubated overnight at 4 °C. For the analyses of the samples from RA cohorts B and C and from baseline, and of the samples from other autoimmune diseases mixed NET preparations from PMA- and A23187-stimulated neutrophils were used. Subsequently, the wells were blocked by incubation for 1 h at room temperature with blocking buffer (5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBST)). After blocking, patient sera were added, 100-fold diluted in blocking buffer, followed by an incubation for 1.5 h at 37 °C. After 3 washings with PBST, the wells were incubated with 100 μl peroxidase-conjugated goat-anti-human immunoglobulin (Dako, Cat. no P0212), 2000-fold diluted in blocking buffer, for 1 h at room temperature. After final washings with PBST (twice) and PBS (twice), the wells were incubated with TMB (Invitrogen, Cat. no 00-4201-56) according to the manufacturer's guidelines. The staining reaction was stopped by adding 2 M H₂SO₄ and the absorbance at 450 nm was measured with a plate reader (Tecan Sunrise).

2.6. Immunofluorescence microscopy

Neutrophils were seeded on microscopic glass slides, NET formation was induced, and the cultures were treated with PMSF as described above. After 3 h 15 m, the slides were washed once with PBS before the cells were fixed with ice-cold methanol for 30 min at –20 °C. After 3 washes with ice-cold acetone, the slides were air-dried and stored at –20 °C until further use. For immunofluorescence, the slides were blocked with blocking buffer (5% non-fat dry milk in PBS containing 0.05% Tween-20 (PBST)) for 60 min at room temperature. Subsequently, the cells/NETs were incubated with 100-fold diluted patient serum in blocking buffer for 1.5 h at room temperature. After three washes with PBST, the cells were incubated with 400-fold diluted ALEXA Fluor® 568-conjugated goat-anti-human IgG antibody (Life Technologies, cat. no. A11004) in blocking buffer for 30 min at room temperature. The slides were washed three times with PBS before staining with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) in PBS for 5 min. After final washings in PBS and Milli-Q water, the slides were covered with coverslips using Mowiol (Sigma Aldrich). Cell staining was analysed on a Leica DMRA Fluorescence

microscope with a Leica DFC340 FX CCD camera.

2.7. Image analysis

Immunofluorescence image analysis was performed semi-automated using FIJI software [22]. To measure the binding of RA antibodies to NETs, a mask of the NET structures was determined by subtracting the mask of all cell bodies from the mask of all the stained structures in the image. The mask of all stained structures in the image was made with a low threshold to get rid of background pixels. The mask of the cell bodies was made with a high threshold to select for cell nuclei or cell bodies still containing high DNA levels. This approach allowed the determination of fluorescence intensities of the NETs (expressed in grey values), which were measured in both the DAPI and ALEXA Fluor 568 channels. As a consequence, the staining of NETs by the RA sera (ALEXA Fluor 568 channel) could be normalized based on the amount of NETs as measured in the DAPI channel.

2.8. Statistical analysis

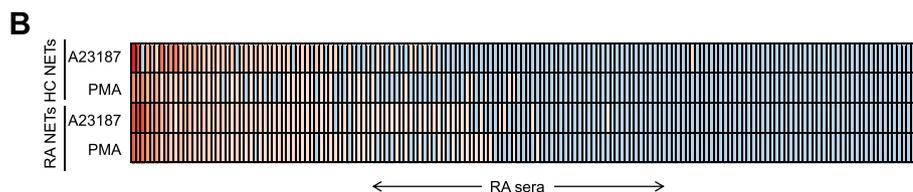
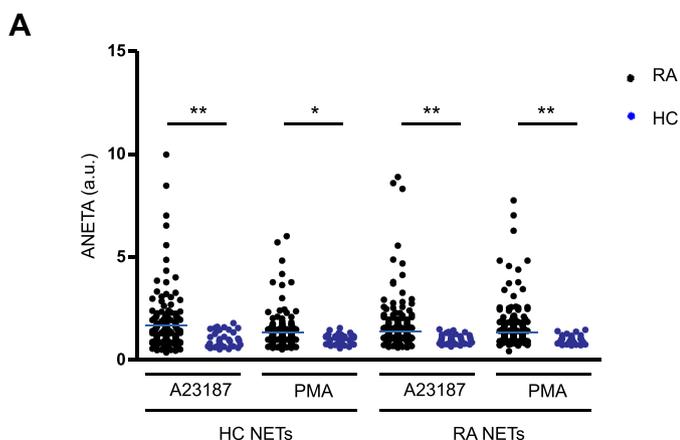
The significance of differences between datasets was determined by a Student's t-test. For correlation analysis, the Pearson r was calculated. Graphs were made and statistical analyses were performed using the GraphPad Prism 7 software (version 7.04, GraphPad Software, Inc).

3. Results

3.1. ELISA analysis of ANETA in RA patient sera

To detect the presence of anti-NET antibodies (ANETA) in sera we developed an ELISA using NET preparations harvested from freshly isolated neutrophils. Since it has been previously reported that NETs induced by different stimuli partially differ in composition [12], we first assessed the reactivity of 168 RA sera (Cohort A) with NETs induced by either A23187 or PMA, using neutrophils from both healthy individuals and RA patients. The immobilized NETs were incubated with 36 healthy control and 168 RA sera to allow the ANETA to bind. The average signals of healthy control sera plus two times the standard deviation were used as cut-off values.

In total, 85 of the 168 RA sera (51%) showed reactivity against at



least one of the NET preparations (Fig. 1A). More than 40% of the RA sera were reactive with NETs from RA neutrophils; 43% reacted with A23187-induced NETs and 39% reacted with PMA-induced NETs. Twenty eight percent of the RA sera were reactive with NETs from healthy individuals, generated either with A23187 or with PMA. Although NETs from healthy controls were somewhat less frequently recognized compared to those from RA patients, this difference did not appear to be statistically significant (p -value = 0.278). The difference between RA reactivity against A23187-induced and PMA-induced NETs almost reached statistical significance (p -value = 0.06). The strong ANETA-positive sera showed reactivity with most if not all NET preparations, whereas the sera that were less strongly reactive recognized only one or a subset of NET preparations (Fig. 1B).

3.2. ANETA in RA patient sera detected by immunofluorescence

To substantiate the results obtained with the ANETA ELISA, we analysed the same sera in an immunofluorescence assay. In brief, neutrophils of RA patients and healthy individuals were seeded on glass slides and stimulated with PMA to form NETs. After fixation of the cells and NETs, the slides were incubated with the RA patient and healthy control sera and antibody-binding was visualized with an Alexa-568-labeled secondary antibody with DAPI used as counterstain. Digital images were recorded by fluorescence microscopy and quantified with a semi-automated approach using FIJI software. The FIJI script differentiates between autoantibody binding to NETs and to cell bodies as described in the Methods section. All samples, 112 RA and 19 healthy control sera, analysed in these immunofluorescent assays (Fig. 2A) were also used in the ANETA ELISA described above. The quantification of NET fluorescence by the FIJI script showed that there was no significant difference between the signals obtained with NETs originating from RA neutrophils and from healthy control neutrophils (data not shown). The FIJI-based quantification data correlated well with the assessment of the same images by eye, indicating that this approach results in an accurate quantification of ANETA (Supplementary Figure 1). In total, the immunofluorescence analyses showed that 48% of the RA patients are ANETA-positive (Fig. 2B). This is similar to the percentage of RA patients' sera that were found to contain ANETA by ELISA (51%, Fig. 1). Indeed, the immunofluorescence scores correlate reasonably well with the ELISA scores (Pearson r = 0.601, p < 0.0001), indicating that both

Fig. 1. ANETA in sera of RA patients. (A) ANETA was measured in 168 RA sera and 36 healthy control sera using ELISA with pooled NET preparations obtained from neutrophils of human controls (HC) and RA patients (RA), which were stimulated by either A23187 or PMA. The reactivity values (a.u.: arbitrary units) were calculated by dividing the absorption of each individual serum by the average of the healthy control sera. Blue bars represent the cut-off values (mean plus 2 times standard deviation of healthy control sera that were analysed in parallel on the same plate). * p < 0.01, ** p < 0.001, Student's t-test. (B) Heatmap of the reactivity of the RA sera with the different types of NETs. Red intensity corresponds to reactivity value, blue indicates no reactivity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

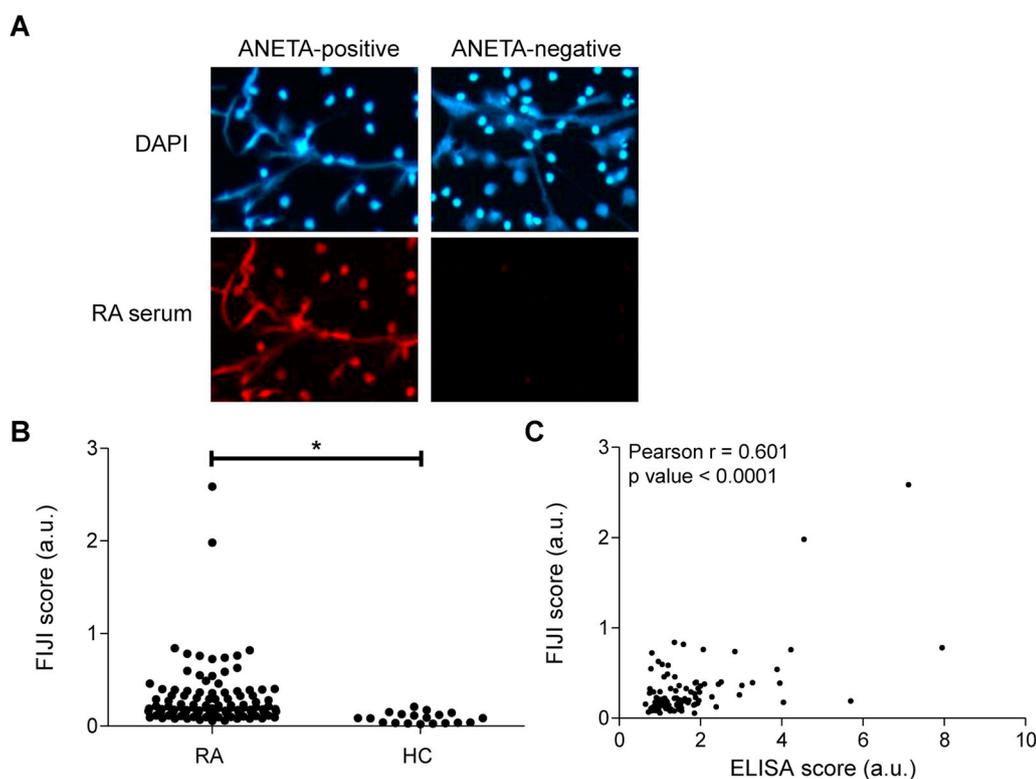


Fig. 2. Reactivity of RA patients' sera with NETs visualized by immunofluorescence and quantified by FIJI. (A) Examples of images representing ANETA-positive and ANETA-negative RA sera. (B) FIJI-based quantification of ANETA in sera of RA patients and healthy controls (HC) ($n = 112$, $*p < 0.01$). (C) Correlation between ANETA detection by ELISA and by immunofluorescence (FIJI score). a. u.: arbitrary units. $n = 112$.

methods can be applied to quantify the reactivity of the RA sera against NETs (Fig. 2C).

3.3. ANETA in other RA patient cohorts and in other autoimmune diseases

To get more insight in the frequency by which ANETA occur in established RA patients, samples from two other RA cohorts (B and C) were analysed by the ANETA ELISA. Also in these RA cohorts ANETA were frequently detected, although the percentages of ANETA-positive samples showed marked differences, 22% and 69%, respectively (Table 1). The combination of all established RA sera tested (Cohorts A, B and C) leads to a frequency of 41%. To investigate whether ANETA are preferentially found in RA sera or occur also in sera from other autoimmune patients, serum samples of patients with systemic lupus erythematosus (SLE), Sjögren's syndrome (SjS) and scleroderma (SSc) were analysed for the presence of ANETA (Table 1). Most ANETA-positive patients were found in the SjS cohort (76%), followed by the SSc cohort (61%). In SLE 36% of the patients was ANETA-positive, but the ANETA-positive SLE patients on average have the highest ELISA scores compared to the other disease groups (Supplementary Figure 2).

3.4. ANETA in newly diagnosed RA patients and after follow-up

It has been well documented that the best characterized

Table 1
 Frequency of ANETA positive patients in different disease cohorts.

	# of patients	# ANETA-positive	% ANETA-positive
Established RA	453	181	40
Cohort A	168	85	51
Cohort B	215	48	22
Cohort C	70	48	69
Early RA	59	41	69
SLE	56	20	36
SjS	59	45	76
SSc	57	35	61

autoantibodies in RA, ACPA and RF, in most cases can be detected already early during disease development. To get more insight in the appearance of ANETA during the course of RA, samples taken at baseline (BL) and after a median follow-up of 7 years (FU; IQR: 6.2–7.9 years) were tested in the ANETA ELISA. ANETA were present both in BL and FU samples (Fig. 3). Both increased and decreased ANETA levels were observed in FU samples as compared to the matching BL samples. No significant differences in ANETA levels between the BL and FU samples were observed.

3.5. Circulating NETs in RA patient sera

The presence of ANETA in the sera of RA patients raised the question whether also NETs or NET fragments were present in these sera. The presence of NET fragments might increase the chance of developing autoantibodies against them. To investigate this possibility, we applied a sandwich ELISA to detect NET fragments in the sera of RA cohort A. An immobilized mouse monoclonal antibody to human myeloperoxidase (MPO) was used to capture MPO-containing complexes in patient sera. Captured NET complexes were detected with a peroxidase-conjugated monoclonal anti-DNA antibody. Variable levels of NET fragments were detected in 32% of the RA sera (Fig. 4A). Interestingly, we did not find a significant correlation between circulating NETs and ANETA (Fig. 4B).

3.6. ANETA and circulating NETs in seropositive and seronegative RA patients

To investigate whether the presence of ANETA or circulating NETs is associated with the autoantibodies often found in RA, RF and ACPA, their co-occurrence was analysed. No association was observed between ANETA and ACPA as measured by the anti-CCP2 test for RA cohort A (Fig. 5A). This suggests that anti-NET reactivity is not or only to a limited extent mediated by the recognition of citrullinated epitopes on NETs. This was corroborated by the results obtained with RA cohorts B & C (Supplementary Figure 3). In contrast, ANETA levels appeared to be

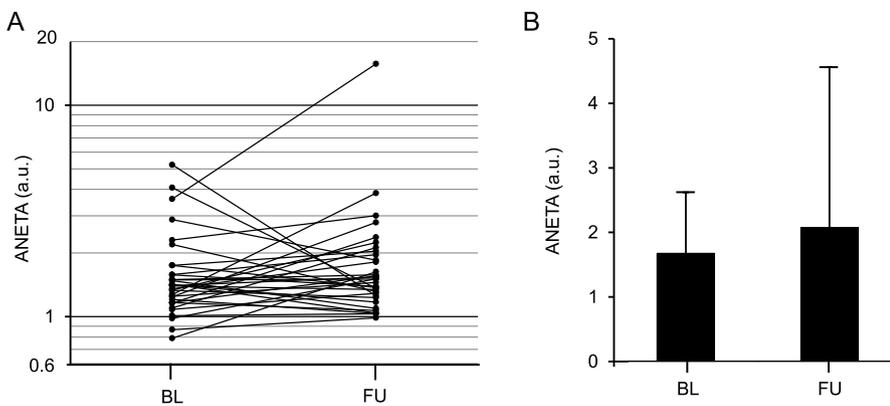


Fig. 3. Presence of ANETA in RA at baseline and after follow-up. (A) Serum was taken from 34 RA patients shortly after diagnosis (BL) and after a median follow-up of 7 years (FU). The presence of ANETA was determined by ANETA ELISA. The reactivity values (a.u.: arbitrary units) were calculated by dividing the absorption of each individual serum by the average of the healthy control sera. (B) Average ANETA reactivities of BL and FU samples (n = 34).

significantly higher in RF-positive than in RF-negative sera (Fig. 5B, $p < 0.001$). No significant association was found between circulating NET levels and either ACPA or RF (Fig. 5C and D). The lack of an association between ANETA and ACPA and between circulating NETs and ACPA suggested that ANETA and/or circulating NETs might be interesting for the classification of the seronegative subgroup of RA patients. However, the frequency by which they occur in this group of patients is relatively low. Only 1 of the 27 seronegative patients has ANETA and circulating NETs were detected in only 4 of these patients.

The association of ANETA with RF might at least in part be explained by the binding of RF to the Fc region of ANETA that have bound their targets on NETs and thereby enhance the signal. Furthermore, RF is known for its non-specific binding and its interference with other laboratory tests that detect antibodies [23]. Since IgM is also the most prominent isoform of RF in RA [24], we repeated our ANETA ELISA by using a secondary antibody that is specific for IgG. We did not find any difference between anti-IgG and anti-IgG, -IgA, -IgM as secondary antibody, suggesting that RF does not yield false positive results in our ANETA assay (data not shown).

3.7. Association of ANETA with demographic and clinical parameters

To gain more insight in the clinical value of ANETA as a biomarker, we investigated their association with clinical features. In addition to age and gender, ANETA levels of ANETA-positive RA patients were compared with disease duration and activity scores (DAS28) and with the general inflammation markers C-reactive protein (CRP) and the

erythrocyte sedimentation rate (ESR). No correlation was observed for any of these features (Fig. 6).

4. Discussion

In this study we have shown that a substantial fraction of RA patients and patients with other autoimmune diseases have antibodies against NETs. ANETA in patient sera were detected both by a newly developed ELISA assay and by immunofluorescence, and a significant correlation between the results obtained with both methods was observed. The reactivity of the RA sera with NETs generated with neutrophils from different donors, RA patients or healthy controls, and produced upon stimulation with PMA or A23187, did not significantly differ. The presence of ANETA weakly correlated with RF, but not with ACPA, circulating NETs in serum, disease progression, disease activity or inflammation markers. This makes ANETA an independent biomarker and additional studies have to reveal whether it might be associated with a clinical subgroup of RA and other autoimmune patients.

To our knowledge, this is the first study that reports the prevalence of ANETA in RA sera. The presence of ANETA in RA sera was reported before, but these studies did not address the frequency by which ANETA occur in RA patients [9,13,25]. Our study indicates that antibodies against NETs are frequently present in RA patients (22%–69% of established RA patients), and often can already be detected at baseline. NETs and ANETA were proposed to contribute to the onset and progression of RA [9], but evidence is lacking. Since NETs are formed in response to pathogens, NET fragments might be cleared by local

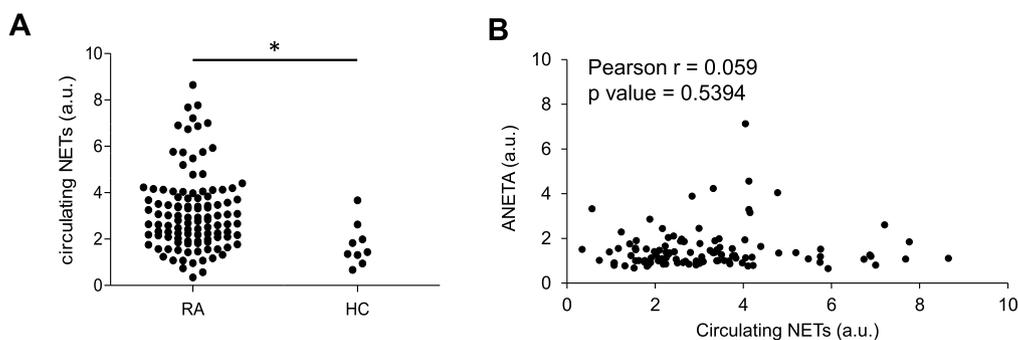


Fig. 4. Circulating NETs in RA patient sera. (A) Using a sandwich ELISA circulating NETs in sera of RA patients (n = 110) and healthy controls (n = 9) were determined. * $p < 0.05$ (B) Correlation between circulating NETs in RA sera and ANETA as measured by ELISA (n = 110).

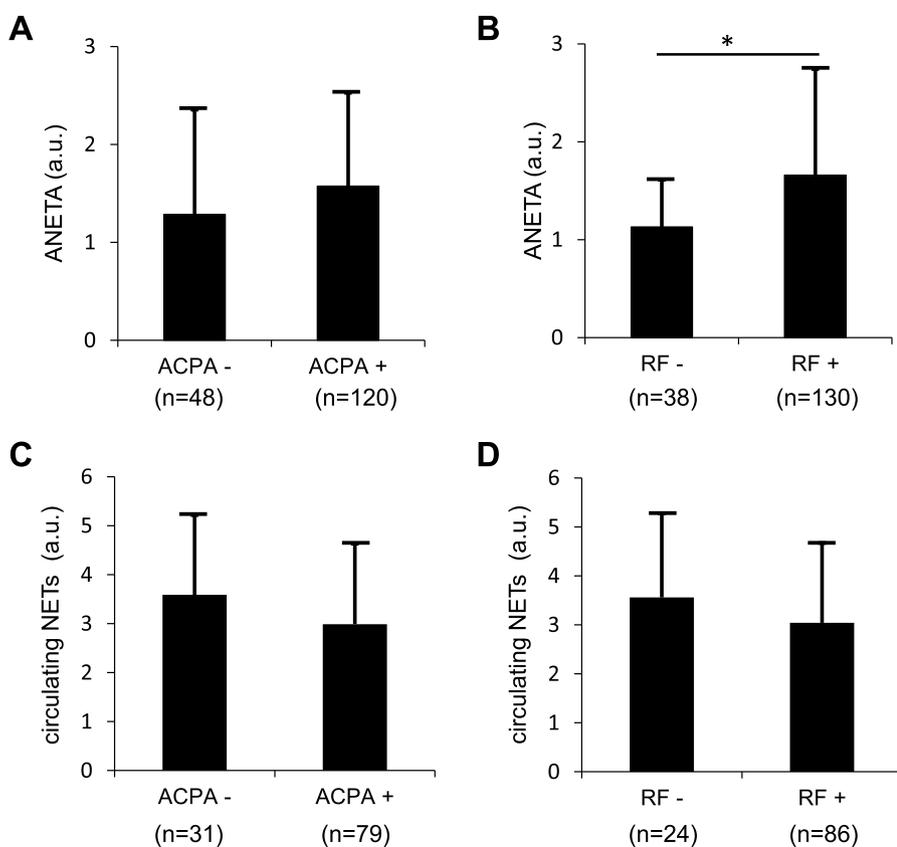


Fig. 5. Association of ANETA and circulating NETs with ACPA and RF. (A) Average ANETA ELISA score for ACPA-negative and positive (anti-CCP2) sera. (B) Average ANETA ELISA score for RF-negative and positive sera. n = 168 *p < 0.001, student's t-test. (C) Average circulating NETs level for ACPA-negative and positive (anti-CCP2) sera. (D) Average circulating NETs level for RF-negative and positive sera. n = 110.

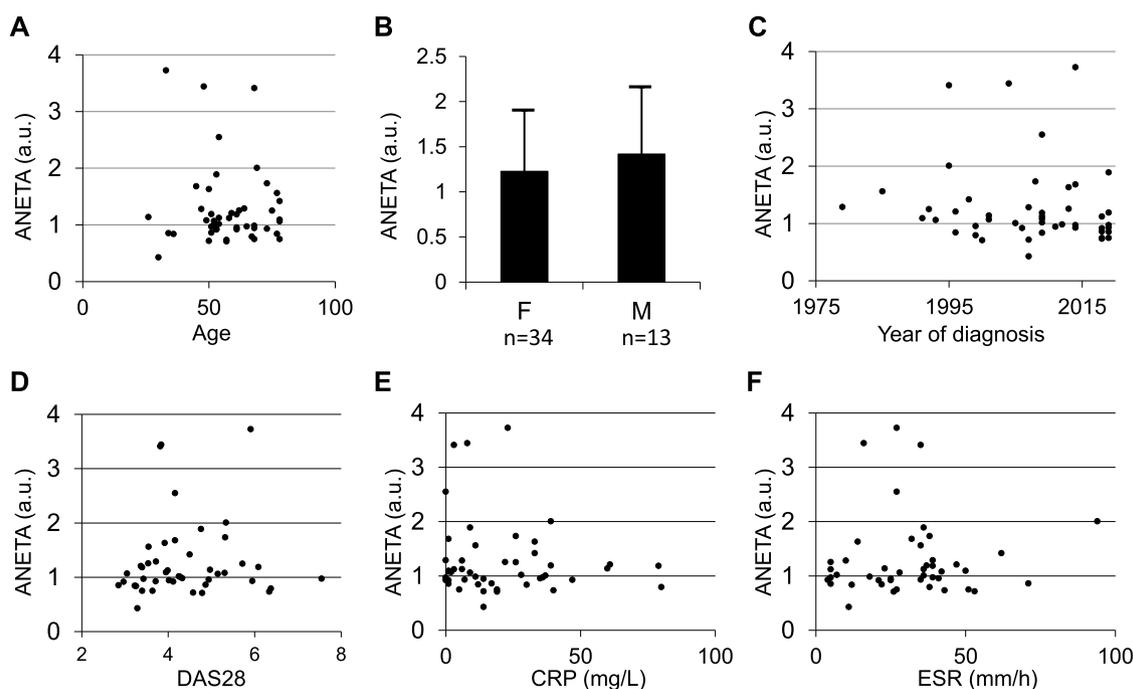


Fig. 6. Correlation analysis between ANETA and demographic/clinical features. The ANETA levels were compared with several demographic and clinical features of the RA patients. (A) Age. (B) Gender (female, F and male, M). (C) Disease duration (year of diagnosis). (D) DAS28. (E) CRP levels. (F) ESR scores. N = 47.

phagocytes together with bacterial remnants, which may act as adjuvants and in that way initiate an autoimmune response against NETs in genetically susceptible individuals [15]. Once the immune response against NETs has been initiated, ANETA might contribute to the vicious cycle of inflammation in RA patients. ANETA bound to NETs may activate the complement system via the classic pathway, leading to an

inflammatory response [26].

We observed variation in the prevalence of ANETA in the three RA cohorts tested, which varied from 22% to 69%. Although these differences might be due to differences in patient sampling and sample storage, it would be interesting to investigate whether therapeutic differences affect the occurrence of ANETA in patients. The analysis of BL

and FU samples of 34 RA patients did not support a common change in ANETA levels during the course of the disease. The elucidation of the underlying cause of the fluctuations observed in a subset of patients requires further research.

We have also assessed the prevalence of ANETA in patients with SLE, SjS and SSc and conclude that the production of ANETA is not specifically associated with RA. The presence of ANETA in other diseases, in particular SLE, has been reported previously by several investigators [21,27]. Although we observed a lower frequency of ANETA in SLE than in SjS and SSc, the reactivity levels appeared to be highest in SLE. Additional studies with larger numbers of patient samples will be required to draw sound conclusions on the prevalence of ANETA in autoimmune, and possibly also other inflammatory diseases. Indeed, ANETA was also demonstrated in microscopic polyangiitis [28].

The epitopes that are recognized by ANETA remain unknown. The results of many studies have shown that NETs contain citrullinated proteins [9,12,13], and therefore, it is likely that at least some of the ANETA represent ACPA. ANETA reactivity in RA sera did not correlate with ACPA reactivity, which strongly suggests that other epitopes play a more prominent role. It is possible that NETs contain multiple epitopes that are recognized by different sera.

Knowledge on the main NET-associated autoepitopes would facilitate the development of a more standardized anti-NET ELISA. The need for fresh human blood samples and the technically challenging procedure to generate NETs imply that independently prepared NETs may show some heterogeneity. Although pooling of NET harvests may at least in part resolve this problem, it is important to note that we did not observe significant differences between the recognition of the NETs of healthy controls and RA patients, nor between that of NETs that were induced by PMA and A23187. Interestingly, Chapman and colleagues showed that the protein composition of NETs induced by PMA and A23187 do show differences [12], which suggests that universal NET components confer their main antigenicity.

We validated our anti-NET ELISA by performing immunofluorescent staining of NETs with the same sera that were tested in ELISA. There is a significant correlation between the results of both methods, substantiating the applicability of the anti-NET ELISA. However, some sera show high reactivity with NETs in immunofluorescence, but have a relatively low ANETA response in the ELISA or vice versa. This might be due to technical differences between both techniques, since immunofluorescent staining of NETs includes a fixation step, whereas the NETs are not fixed in the ELISA. Fixation might affect the accessibility and the structure of epitopes.

Interestingly, ANETA do not correlate with the presence of NET fragments in serum. The co-occurrence of ANETA and NET fragments in the sera of some patients raises the question as to what extent ANETA and NETs exist as immune complexes and to what extent such immune complexes affected their detection in the assays applied. ANETA binding to NETs in serum may interfere with their binding to NETs in the ANETA ELISA and immunofluorescence assays. As a consequence, the ANETA levels detected may be an underestimation of the actual levels. Similarly, ANETA-binding to circulating NETs may reduce the levels of NETs that can be detected in sera. Further studies are needed to clarify this issue.

5. Conclusions

In this study, an anti-NET ELISA was developed that can be used for the detection of ANETA in human serum. A substantial fraction of both early and established RA patients appeared to have antibodies against NETs in their serum and about one third of the patients have detectable levels of NET (-fragment)s. ANETA are not specific for RA, but are also found in sera from patients with other autoimmune diseases. ANETA were significantly higher in those patients who were RF positive, but ANETA were not associated with ACPA or any other clinical parameter. Further research is needed to investigate the clinical value of ANETA

and circulating NET(-fragment)s.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaut.2020.102484>.

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